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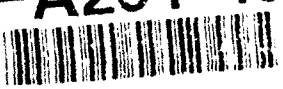

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We have utilized polymerase chain reaction with primers corresponding to conserved amino acid sequences within membrane-spanning regions of known serotonin receptors to identify clones of 4 putative new indoleamine receptors. We have determined complete amino acid sequences of these 4 receptors which fall into 3 subfamilies; two of these subfamilies are novel. The sites of expression within the brain have been determined for each of the genes. Expression in mammalian cells demonstrates that each new protein is a receptor for serotonin and each has a distinct pharmacology when compared to known receptors. Two of the new receptors are coupled to CAMP, one negatively (G) and one positively (Gs). The latter is a candidate for the serotonin receptor that mediates phase advances in circadian rhythms of the SCN.

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MOLECULAR APPROACH TO HYPOTHALAMIC RHYTHMS:

ISOLATION OF NOVEL INDOLEAMINE RECEPTOR GENES

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ABSTRACT

We have utilized polymerase chain reaction with primers corresponding to conserved amino acid sequences within membrane-spanning regions of known serotonin receptors to identify clones of 4 putative new indoleamine receptors. We have determined complete amino acid sequences of these 4 receptors which fall into 3 subfamilies; two of these subfamilies are novel. The sites of expression within the brain have been determined for each of the genes. Expression in mammalian cells demonstrates that each new protein is a receptor for serotonin and each has a distinct pharmacology when compared to known receptors. Two of the new receptors are coupled to cAMP, one negatively (G_i) and one positively (G_s). The latter is a candidate for the serotonin receptor that mediates phase advances in circadian rhythms of the SCN.

The phase of endogenous circadian rhythms are modulated by the indoleamine neurohormonal transmitters (5-hydroxytryptamine; 5-HT) and melatonin (Cassone, 1990; Krause and Dubocovich, 1990; Medanic and Gillette, 1992; Edgar et al. in press; Miller and Fuller, 1990 and Prosser et al. in press). Biochemical studies indicate that the mechanism by which 5-HT and melatonin cause phase shifts in the suprachiasmatic circadian pacemaker is in part mediated by G-protein-associated receptors (Carlson, et al., 1987; Medanic and Gillette, 1992; Prosser et al., in press). Application of 5-HT to the *in vitro* suprachiasmatic nucleus (SCN) preparation (reviewed by Gillette, 1991) induces phase advances in spontaneous neuronal activity during subjective day; pharmacological studies indicate that 5-HT exerts this action via a 5-HT_{1A} or 5-HT_{1A}-like receptor (Medanic and Gillette, 1992; Prosser et al., in press).

Genes encoding several subtypes of serotonin (5-HT) receptors have been cloned, and except 5-HT_3, all encode G-protein-associated molecules that span the plasma membrane seven times (Albert et al., 1990; Voigt et al., 1991; Julius et al., 1988; Hamblin and Metcalf, 1991; Hartig et al., 1992; McAllister et al., 1992; Prichett et al., 1988; Lubbert et al., 1987). No gene encoding a melatonin receptor has yet been isolated, however there is a melatonin receptor in the SCN (Vanecek, 1988) and melatonin binding to its receptor has been shown to mediate pertussis toxin-sensitive inhibition of cAMP accumulation (Carlson et al., 1989), suggesting that the receptor is a G-

protein coupled protein from the same protein super family. These data suggested to us that a study of molecules expressed within the SCN that are related to serotonin receptors might lead to identification of proteins involved in the mediation of indoleamine signaling in relation to rhythmicity and also suggested a molecular strategy that would allow their identification.

STRATEGY

To isolate clones of indoleamine-binding receptors belonging to the superfamily of G-protein-associated receptors, we refined the strategy reported by Libert et al. (1989), who demonstrated that clones of novel members of this superfamily could be isolated through the use of degenerate primers corresponding to conserved regions of transmembrane domains (TMDs) III and VI in a polymerase chain reaction (PCR). We have extended this approach by performing two sequential rounds of nested PCR on a rat hypothalamic cDNA template (hypothalami dissected during mid-subjective day and mid-subjective night of rats entrained to a 12:12 L/D cycle) using degenerate primers in the second round of PCR that correspond to conserved residues specific to indoleamine-binding receptors. Our strategy increases the probability of isolating only indoleamine binding-receptors and amplifying indoleamine-binding receptors encoded by mRNAs of low abundance, and decreases the probability of cloning artifactual cDNAs.

Two different variations of this general strategy were used to isolate clones of four novel indoleamine-binding receptors designated as MR77, MR22, REC17 and REC20. The specific design which led to the subsequent cloning of MR77, MR22 and REC17 from hypothalamic cDNA was a first round of PCR using degenerate primers corresponding to conserved regions in TMDs III and VI of both catecholamine and 5-HT receptors. This was followed by a second round of PCR using a degenerate primer corresponding to a conserved region of TMD V, specific only to 5-HT receptors, in conjunction with the same TMD III primer used in the first round of PCR (Lovenberg et al., 1992; Erlander et al., 1992).

For isolating REC20, we performed the first round of PCR - mediated hypothalamic cDNA amplification (here we used cDNA from rat cortex and striatum as well) using degenerate primers corresponding to regions of TMD III and TMD VII conserved among catecholamine and 5-HT receptors. The second PCR round used a degenerate primer corresponding to residues in TMD V specific to MR22 and REC17 in conjunction with the already-used TMD VII primer (Lovenberg et al., submitted).

SEQUENCE ANALYSIS

By performing these amplification strategies we obtained PCR-derived cDNAs and used them as probes to screen a rat hypothalamus cDNA library (MR22, REC17 and REC20) or rat genomic library (MR77) to obtain clones spanning the entire protein coding regions. We determined their nucleotide sequences and

found that each putatively encoded a protein with the amino acid sequence characteristic of members of the G-protein-coupled, seven-TMD receptor superfamily. A search of the protein and nucleic acid databases with the primary structures of these four putative receptors revealed that MR77 has significant amino acid sequence identity with known 5-HT receptors that are coupled to the inhibition of adenylate cyclase: human 5-HT_{1E} = 55%, dog 5-HT_{1D} = 48% and rat 5-HT_{1B} = 46%. In contrast, MR22, REC17 and REC20 have 30-35% amino acid sequence identity with catecholamine and 5-HT receptors alike, with none exhibiting dominant similarity. However, MR22 and REC17 have 68% mutual sequence identity. These observations suggest that MR77 is a member of the 5-HT_{1E} subfamily, that MR22 and REC17 form a new subfamily, and that REC20 falls into a subfamily by itself (Lovenberg et al., 1992; Erlander et al., 1992; Lovenberg et al., submitted).

mRNA DISTRIBUTIONS

We performed Northern blots with rat poly(A) selected RNA extracted from eight brain regions (cortex, hypothalamus, thalamus, hippocampus, striatum, pons, medulla and cerebellum) and heart, liver and kidney (Lovenberg et al., 1992; Erlander et al., 1992; Lovenberg et al., submitted). MR22 mRNA was detected only in the hippocampal sample. REC17 mRNAs were detected in hippocampus > cortex = thalamus = pons = striatum = medulla. REC20 mRNAs were found in hypothalamus = thalamus > pons = hippocampus. MR77 was not detected in any of the tissues

examined by this method, however, by using a semi-quantitative PCR technique we found MR77 mRNAs in cortex = striatum = hippocampus > thalamus = pons > hypothalamus > cerebellum. None of the receptor mRNAs was detected in heart, liver or kidney.

MR22, REC17, MR77 AND REC20 ARE ALL 5-HT RECEPTORS

To determine the ligand(s) for these four putative receptors empirically, we subcloned their cDNAs into a eucaryotic expression vector and transiently expressed the encoded protein is CosM6 cells. We found that broken cell preparations from transfected cells containing either MR22, REC17, MR77 or REC20 exhibited saturable binding for [^{125}I]-LSD whereas mock-transfected cells exhibited no measurable [^{125}I]-LSD binding (Lovenberg et al., 1992; Erlander et al., 1992; Lovenberg et al., submitted). ^{125}I-LSD has been shown previously to be a non-selective serotonergic ligand with high affinity (K_D < 10nM) for all known 5-HT receptors except 5-HT$_{1B}$ (K_D < 10-1000 nM; Peroutka, 1990). The calculated equilibrium dissociation constant (K_D) for the individual receptors is as follows: MR22 = 2.0 nM, REC17 = 0.64 nM, MR77 = 14 nM and REC20 = 1.5 nM. We next tested the ability of several biogenic amine neurotransmitters to displace [^{125}I]-LSD binding from these receptors. Serotonin was able to displace [^{125}I]-LSD binding to all receptors; neurohormones melatonin, dopamine and epinephrine had no measurable effects.

PHARMACOLOGICAL PROFILE AND CLASSIFICATION

To relate each of these receptors to known members of the 5-HT receptor family, we performed three analyses. We determined the ability of selective 5-HT agonists and antagonists to displace LSD from the membrane preparations. We compared their primary structures with other known 5-HT receptors by a dendrogram analysis (figure 1). We also expressed each receptor in HeLa cells to determine its ability to mediate cAMP accumulation.

[^{125}I-LSD] binding to the MR77 protein is sensitive to sumatriptan, a 5-HT_{1D} agonist, but insensitive to 5-carboxyamidotryptamine (5-CT), a mixed 5-$HT_{1A/1D}$ agonist, and 8-OH-DPAT, a 5-HT_{1A} agonist (Lovenberg et al., 1992). The pharmacological profile of MR77 is similar to that of the 5-HT_{1E} receptor. Because MR77 has greatest identity (55%) with a recently cloned 5-HT_{1E} receptor we conclude that we have identified a second member of the 5-HT_{1E} family and have designated MR77 as the 5-HT_{1F} receptor. This relationship is illustrated in the dendrogram shown in figure 1; as indicated, MR77 belongs to the 5-HT_1 (5-$HT_{1A/B/D/E}$) family. In functional terms, we have demonstrated that MR77 (5-HT_{1F}) mediates the inhibition of cAMP accumulation in HeLa cells, presumably through a G_i-protein.

MR22 and REC17 have similar pharmacological profiles which do not readily fit to any pharmacologically defined 5-HT receptors which have been previously described (Erlander et al.,

1992). Both receptors are sensitive to ergotamine, 5-CT and methiothepin but insensitive to sumatriptan and 8-OH DPAT. This is consistent with their primary structural relationships with other known 5-HT receptors. As shown in the dendrogram, the primary structures of MR22 and REC17 lie in between those of the 5-HT_1 and 5-HT_2 (5-HT_{1C} and 5-HT_2) families. We were unable to link either MR22 or REC17 to a functional response; neither receptor mediated stimulation nor inhibition of cAMP accumulation in HeLa cells. MR22 and REC17 may couple to a second messenger other than cAMP. We propose that MR22 and REC17 are two members of a third subfamily of 5-HT receptors.

REC20 has a pharmacological profile that is unique (Lovenberg et al, submitted). Displacement of [^{125}I]-LSD binding to REC20 is sensitive to 5-HT_{1A} agonists 8-OH DPAT and 5-CT but also sensitive to the 5-HT_2 antagonist ritanserin. Comparison of the REC20 primary structure with other known 5-HT receptors indicates it forms a fourth subfamily (figure 1). REC20 stimulates the accumulation of cAMP in HeLa cells. Thus, unlike all other known 5-HT receptors, REC20 couples to a G_s-protein.

REC20 IS A CANDIDATE FOR MEDIATING 5-HT-INDUCED PHASE ADVANCES

We examined the mRNA expression patterns, pharmacological profiles and functional properties of each receptor to determine whether these qualities matched properties of 5-HT receptors previously identified as being involved in SCN-clock mechanisms. In Table I, we summarize what is known about MR77, MR22, REC17

and REC20. REC20 is a particularly good candidate for the receptor mediating 5-HT induced phase advances in the SCN given that Medanic and Gillette (1992) have demonstrated in an *in vitro* preparation of the SCN that a 5-HT-induced phase shift in electrical activity during subjective day is mediated by a 5-HT receptor that potently binds 8-OH-DPAT and 5-CT. In addition, these workers have shown that an increase in the cAMP concentrations during the same window of time during subjective day results in a similar phase shift. REC20 is an appealing candidate for the relevant receptor because it binds these 5-HT_{1A} agonists, but unlike $5HT_{1A}$, is coupled to increases in cAMP. Future studies are directed toward determining whether REC20 mRNA is present in the SCN (it is expressed in the hypothalamus, whereas 5-HT_{1A} mRNAs were undetectable in the SCN by *in situ* hybridization; Roca et al., 1992) followed by use of pharmacological tools to discriminate functional responses mediated by REC20 and 5-HT_{1A} receptors within the *in vitro* SCN preparation.

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Table 1. Summary of novel 5-HT receptor characteristics. ORF refers to the triplet length of the open reading frame encoding the amino acid sequence. mRNA distribution was determined by Northern blot analysis (CX, cortex; STR, striatum; HIP, hippocampus; HYP, hypothalamus; THAL, thalamus; PONS, pons; MED, medulla). 2nd messenger coupling refers to the ability of the receptors to activate and inhibit adenylate cyclase through stimulatory (G_s) or inhibitory (G_i) G-proteins, respectively. The coupling of two receptors is unknown (?). Sequence similarity was determined by comparison of the complete amino acid sequences to the receptors listed in the table. K_i values represent the concentration of [^{125}I]-LSD required to half saturate receptors transfected in CosM6 cells. The pharmacological classification indicates similarity in the binding profile to those of known serotonin agonists and antagonists.

Figure 1. Dendrogram of known 5-HT receptor subfamilies. This dendrogram represents the pairwise amino acid sequence comparisons of all 5-HT receptors. The lengths of the horizontal bars are inversely proportional to the percent similarity of related sequences. For example, REC17 and MR22 are more similar to each other than any other pair of 5-HT receptors. REC20 and 5HT1A are sufficiently unique in sequence to form independent branches of the 5-HT family.

Characterization of Novel 5-HT Receptor Clones

	MR77	MR22	REC17	REC20
ORF (aa)	366	371	357	391
mRNA distribution	CX, STR, HIP HYP, THAL, PONS	HIP	HIP, CX, PONS, HYP STR, THAL, MED	HYP, THAL, PONS HIP
2nd messenger	G_i	?	?	G_s
Greatest sequence similarity	5-HT_{1E} (55%)	REC17 (68%)	MR22 (68%)	Unique (30% to all GPCRs)
K_i (nM) (^{125}I-LSD)	14	2	0.64	1.5
Pharmacological classification	5-HT_{1E} family	Unique	Unique	5-HT_{1A}-like

Table 1

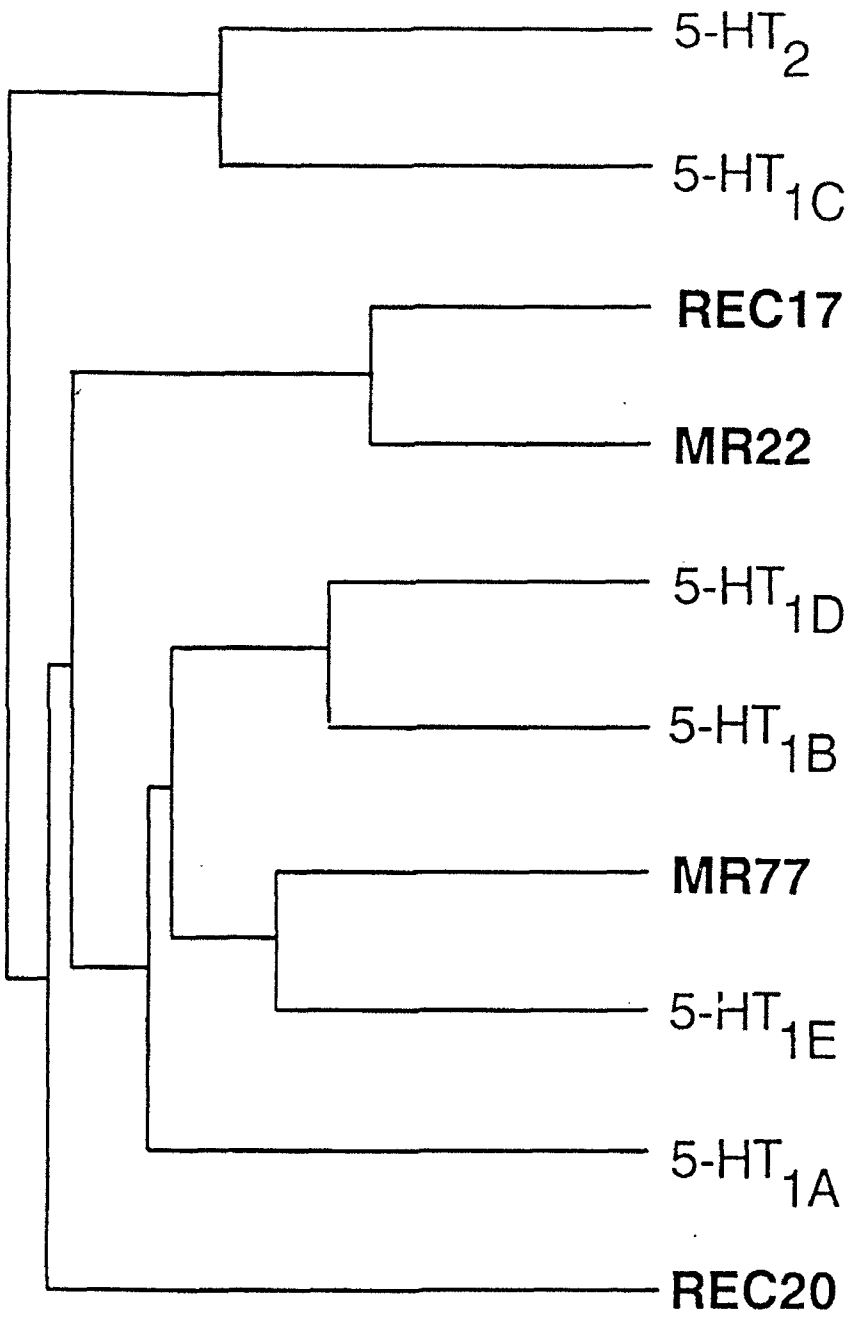

Figure 1

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Neurobiology

Molecular cloning and functional expression of 5-HT_{1E}-like rat and human 5-hydroxytryptamine receptor genes

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ABSTRACT **Sequential polymerase chain reaction experiments were performed to amplify a unique sequence representing a guanine nucleotide-binding protein (G-protein)-coupled receptor from rat hypothalamic cDNA. Degenerate oligonucleotides corresponding to conserved amino acids from transmembrane domains III, V, and VI of known receptors [5-HT_{1A}, 5-HT_{1C}, and 5-HT_2; 5-HT is serotonin (5-hydroxytryptamine)] were used as primers for the sequential reactions. The resulting product was subcloned and used to screen a rat genomic library to identify a full-length clone (MR77) containing an intronless open reading frame encoding a 366-amino acid seven-transmembrane domain protein. The human homolog was isolated, and its encoded protein had 93% overall amino acid identity with the rat sequence. Within the conserved transmembrane domains, the sequences exhibit approximately 52%, 59%, 65%, and 68% amino acid identity with the known rat 5-HT_{1A}, rat 5-HT_{1B}, rat 5-HT_{1D}, and human 5-HT_{1E} receptors, respectively. MR77 was subcloned into a eukaryotic expression vector system and expressed in CosM6 cells. Studies on broken cell preparations indicate that the expressed receptor exhibits ^{125}I-labeled *d*-lysergic acid diethylamide (LSD) binding that can be displaced by serotonin but not by other biogenic amines. The specific binding is displaced by the selective 5-HT_{1D} agonist sumatriptan but not by the mixed 5-$HT_{1A/1D}$ agonist 5-carboxyamidotryptamine. ^{125}I-labeled LSD binding was competitively antagonized by the ergot alkaloids methysergide and ergotamine. HeLa cells transfected with the MR77 gene exhibited inhibition of adenylate cyclase in response to serotonin. MR77 is expressed at low levels throughout the brain, with the greatest expression in the cortex, hippocampus, and striatum. MR77 thus represents a 5-HT receptor of the 5-HT_1 class, and we propose that, based on the pharmacological characterization, MR77 represents an additional 5-HT_{1E}-like receptor.**

Serotonin (5-hydroxytryptamine, 5-HT) is a biogenic amine neurotransmitter found in both the central and peripheral nervous systems (for review, see ref. 1). A majority of serotonin-containing neurons in the central nervous system have cell bodies located in association with the raphe nuclei in the midline area of the brainstem. These cells send projections to the cortex, several limbic structures (hypothalamus, hippocampus, basal ganglia, and amygdala) as well as the lateral geniculate and superior colliculus. Serotonergic transmission through these pathways is thought to be involved with a variety of behaviors and disorders including anxiety, sleep regulation, aggression, and depression. To understand the effects of current drug therapies and also develop additional selective drugs, the receptor systems involved in serotonergic transmission along these pathways must be elucidated.

Pharmacological experiments have suggested that the actions of serotonin are mediated by multiple types of serotonin receptors, each having a distinct profile of activity with respect to serotonin-related drugs (2). Several serotonin receptors have been identified by cDNA cloning and have been shown to be guanine nucleotide-binding protein (G-protein)-coupled molecules with the putative seven-transmembrane domain structure characteristic of many receptors, including the prototypes rhodopsin and the β-adrenergic receptors (3). Members of the serotonin receptor family have been subdivided on the basis of either affinities for serotonin or sequence similarities. Members of the 5-HT_1 subgroup are characterized by their high affinities for serotonin, whereas 5-HT_2 receptors exhibit low affinity. Currently, five 5-HT_1 and one 5-HT_2 receptor have been identified pharmacologically (5-HT_{1A}, 5-HT_{1B}, 5-HT_{1C}, 5-HT_{1D}, 5-HT_{1E}, and 5-HT_2). Genomic and/or cDNA clones for all of these receptors have been isolated, and their sequences have been determined (4–11).

In an attempt to identify other receptors that belong to the family of 5-HT receptors, we used a strategy based upon similarities shared among known members of the family. We designed highly degenerate oligonucleotides corresponding to conserved amino acid sequences in putative transmembrane domains III, V, and VI of the 5-HT_{1A}, 5-HT_{1C}, and 5-HT_2 receptors. These oligonucleotides were used in sequential polymerase chain reactions (PCR) with rat hypothalamus cDNA as the template to generate a series of clones. Here we report that one of these clones and its human homolog¶ correspond to a 5-HT receptor that is more closely related in sequence to the recently cloned human S31 receptor (5-HT_{1E}) than any previously described 5-HT receptor but clearly is a new member of the 5-HT_1 receptor family. Initial pharmacological characterization of this receptor, which is negatively coupled to adenylate cyclase, is similar to but distinct from that described previously for the 5-HT_{1E} receptor (9, 12, 13), and therefore we propose that MR77 belongs to a new subgroup of 5-HT_{1E}-like receptors.

MATERIALS AND METHODS

PCR Cloning, Library Screening, and Sequence Analysis. Poly(A)-enriched RNA was isolated from dissected rat hypothalami using Micro-FastTrack kits (Invitrogen, San Diego) and used as template for first-strand synthesis of cDNA by Moloney murine leukemia virus reverse transcriptase

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Abbreviations: 5-HT, 5-hydroxytryptamine (serotonin); 5-CT, 5-carboxyamidotryptamine; LSD, *d*-lysergic acid diethylamide.

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¶The sequences reported in this paper have been deposited in the GenBank data base (accession nos. L05596 for the rat clone and L05597 for the human clone).

(Stratagene). Oligonucleotides of degenerate sequence were synthesized corresponding to conserved amino acid sequences of portions of putative transmembrane domains III, V, and VI of the 5-HT_{1A} and 5-HT_{1C} receptors [Leu-Cys-Ala-Ile-(Ala or Ser)-Leu-Asp-Arg-Tyr, Phe-(Val or Gly)-Ala-Phe-(Phe or Tyr)-Ile-Pro-Leu, and (Cys or Met)-Trp-(Leu or Cys)-Pro-Phe-Phe-Ile, respectively]. The first PCR reaction (30 cycles of 94°C for 15 sec, 55°C for 30 sec, and 72°C for 30 sec with a Perkin Elmer Cetus 9600 thermal cycler) used oligonucleotides corresponding to transmembrane regions III and VI (10 μg each) with the hypothalamus cDNA as a template. Products >500 bases were isolated by agarose gel electrophoresis and used as templates for a second PCR reaction with oligonucleotides III and V. The resulting products were ligated into pBluescript (Stratagene) and used to transform DH5α bacteria (BRL). Cloned DNA was used to probe both rat and human genomic libraries (Stratagene). All sequencing was performed by the dideoxy-termination method (14) with Sequenase (United States Biochemical). Sequence data were compiled and analyzed by using Genetics Computer Group sequence software (Madison, WI) (15). Nucleotide sequences of primers (*5' to 3'* with N = A, T, G, or C; Y = T or C; H = T, C, or A; W = A or T; S = C or G; R = A or G; M = A or C; D = A, G, or T; and parentheses = different nucleotides in two degenerate sets of oligonucleotides used for primers III and VI) were as follows: III, T-N-T-G-Y-G-C-N-A-T-H-G(W)-C(S)-N-Y-T-N-G-A-Y-M; V, N-A-R-N-G-G-D-A-T-R-W-A-R-A-A-N-G-C-N-M-C-R-A-A; and VI, D-A-T-R-A-A-R-A-A-N-G-G-N(R)-A(C)-R(A)-C-C-A-R(C)-C(A)-A(T).

Expression in COS-M6 Cells. All expression and pharmacological experiments were carried out with the rat MR77 clone. CosM6 cells (a subclonal line of COS-7 cells, obtained from Edith Womack in the laboratory of J. L. Goldstein, University of Texas Health Sciences, Dallas, and generously provided by L. E. Limbird, Vanderbilt University) or HeLa cells were transfected by using the DEAE dextran or calcium phosphate methods, respectively, with plasmids (10 μg of cesium chloride-purified plasmid per 10-cm dish) pDP5HT1a (16), pDP5MR77, or pBC12BIBeta2 (17) (hamster β_2-adrenergic receptor gene). The hamster β_2-adrenergic receptor gene, obtained from ATCC (deposited by R. J. Lefkowitz, Duke University), was subcloned into the pBC12BI eukaryotic expression vector [provided by B. Cullen, Duke University (18)]. Cotransfection experiments used 2 μg of pBC12BIBeta2 combined with 10 μg of either pDP5HT1a (5-HT_{1A}) or pDP5M77 (MR77).

^{125}I-Labeled *d*-Lysergic Acid Diethylamide (LSD) Binding. Approximately 60 hr after transfection, CosM6 cells were removed from dishes by using EDTA (GIBCO) and a cell scraper and then were collected by centrifugation (250 × *g*, 5 min). Pellets were resuspended in 50 mM Tris·HCl (pH 7.6) with a polytron homogenizer, centrifuged for 20 min at 40,000 × *g*, and resuspended in the same buffer (500 μl per 10-cm dish). ^{125}I-LSD (NEN, 2200 Ci/mmol; 1 Ci = 37 GBq) binding was assessed in a 100-μl final volume and contained 0.02% ascorbate, CosM6 membranes (15 μg of protein), 1 nM ^{125}I-LSD (final concentration), and various unlabeled test compounds. Nonspecific binding was determined by using 100 μM serotonin, and typically was <10% of total dpm. After 60 min at 37°C, 2 ml of ice-cold 50 mM Tris·HCl was added to each tube, and bound ligand was isolated on 0.1% polyethyleneimine-soaked GF/B glass fiber filters, washed twice, and assayed for radioactivity. Protein concentration was measured by the Bradford dye binding method (Bio-Rad) (19). Binding data were analyzed by nonlinear regression analysis as described (20).

Cyclic AMP Accumulation. cAMP accumulation was measured by a method similar to that described by Baron and Siegel (21). HeLa cells were transfected by the calcium phosphate method and utilized 64 hr later. The cells were washed twice with 10 ml of Krebs–Ringer buffer (KRB) containing 134 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 2.5 mM $MgSO_4$, 1.18 mM KH_2PO_4, 11.1 mM glucose, 20 mM Hepes·NaOH, and 0.001% phenol red (pH 7.35). KRB containing 4 μCi of [^{3}H]adenine (10 ml per dish) was added, and the dishes were placed in a 37°C air incubator for 2 hr. The dishes were washed once with KRB and once with 10 ml of EDTA, and then the cells were incubated with 10 ml of fresh EDTA for 30 min at 37°C. Cells were resuspended, pooled, and pelleted at 250 × *g* for 5 min. The final pellet was suspended in KRB containing 0.5 mM isobutylmethylxanthine (IBMX) and 0.02% ascorbate, and 400 μl of the cell suspension was dispensed to each assay tube. After a 5-min preincubation at 37°C, the tubes were cooled on ice, various test compounds or KRB/IBMX was added, and the tubes were returned to the 37°C bath for 10 min. The 37°C incubations were ended by placing the tubes on ice and adding 550 μl of ice-cold 10% perchloric acid containing 5500 dpm of [^{14}C]cAMP, 110 μg of unlabeled ATP, 55 μg of unlabeled cAMP, and 0.002% phenol red. Samples were centrifuged at 3700 rpm for 15 min, and the transferred supernatants were neutralized with 50 μl of 10 M KOH. Insoluble $KClO_4$ was removed by centrifugation, and 50-μl aliquots were removed for determination of total radioactivity in each sample. The remaining 1.0 ml was used for determination of cAMP by the sequential Dowex/alumina ion-exchange method (22). Conversion of cell-associated [^{3}H]ATP to [^{3}H]cAMP was expressed as a percentage of total cell-associated radioactivity (from the 50-μl sample) and was normalized to the percentage recovery of [^{14}C]cAMP tracer (generally 25–40%).

Semiquantitative PCR. To determine the tissue distribution of MR77 mRNA expression in rat, we designed a PCR protocol that takes advantage of the intron present in the genomic MR77 clone (Fig. 3*A*). Two oligonucleotide primers were designed—one was the inverse complement of nucleotides 590–615, and the other corresponded to the region of the cDNA 20 nucleotides upstream from the putative splice site. In a PCR assay, these primers amplify a 563-bp fragment from cDNA template but not from genomic DNA. Poly(A)$^+$ RNA was isolated from a variety of dissected rat tissues including several brain regions. First-strand cDNA was synthesized from 1 μg of poly(A)$^+$ RNA from each tissue by using Moloney murine leukemia virus reverse transcriptase. Two microliters of a 1:10 dilution of each cDNA was used as template in a PCR reaction with the primers described above for 30 amplification cycles; 30 cycles was determined to be within the linear amplification range under the conditions used. Equal aliquots (10 μl) of each PCR reaction were subjected to agarose gel electrophoresis. The resulting gel was denatured, neutralized, transferred to nitrocellulose membrane, and probed with a radiolabeled oligonucleotide complementary to nucleotides 364–385 of MR77.

RESULTS AND DISCUSSION

Identification of cDNA and Genomic MR77 Clones. We used sequential amplification of rat hypothalamus cDNAs with nested primers and PCR to identify novel 5-HT receptor-like sequences. The first amplification was performed with degenerate primers (with cloning sites) corresponding to portions of the coding regions of putative transmembrane domains III and VI that are conserved among known catecholamine and serotonin receptors from the G-protein-associated receptor superfamily. Products longer than 500 base pairs (bp) were isolated and used as templates in a second amplification designed to amplify cDNAs encoding proteins closely related to known 5-HT receptors (by using the same transmembrane III degenerate primer and a degenerate primer corresponding to residues in transmembrane V that

are conserved among known 5-HT receptors but not catecholamine receptors). The PCR products were ligated to vector, and bacterial transformation resulted in approximately 1000 colonies, of which >95% hybridized to a mixture of radiolabeled oligonucleotides corresponding to nonconserved regions located between transmembrane domains III and V of the rat 5-HT_{1A} (nucleotides 1002–976), 5-HT_{1C} (nucleotides 879–856), and 5-HT_2 (nucleotides 1515–1488) receptors. Thirty six colonies that showed either minimal or no hybridization were picked and reprobed with the same oligonucleotide mixture, and partial sequences of nonhybridizing clones were determined. From this screening, two distinct cDNA clones were isolated whose sequences had significant similarity to G-protein-associated receptors. One of these, designated MR77, is described in this paper.

We tried unsuccessfully to screen a rat hypothalamus cDNA library with the MR77 PCR product as a probe. Since many neurotransmitter receptor genes of this superfamily do not contain introns, we hypothesized that a genomic clone may contain an intact open reading frame. A rat genomic library was probed with radiolabeled MR77 DNA. This resulted in the isolation and subsequent subcloning of a 2.5-kilobase (kb) fragment that contained an apparently intronless open reading frame of 1098 nucleotides. However, the reading frame upstream from an apparent initiation codon was also open. Therefore, we used an anchor-PCR protocol with an oligonucleotide primer complementary to nucleotides 311–289 and a poly(dC) oligonucleotide to amplify template rat brain cDNA tailed with dG and cloned the largest product. The sequence of this partial MR77 cDNA clone was identical to the genomic clone from nucleotide 97 to nucleotide 311 but was completely different at its 5′ end beginning 41 nucleotides upstream from the presumed initiation codon. The cDNA sequence contained stop codons in frame with the putative coding sequence, suggesting that the assignment of the initiation codon was correct. Upon closer examination of the genomic clone, it was evident that an intron acceptor site was at nucleotide 97 (asterisk at top of Fig. 1). The intron lies upstream from the complete open reading frame encoding the putative receptor. Because the open reading frame encoded a 366-amino acid sequence that is highly related to known receptors throughout its entire length, we concluded that the mRNA contained no further introns.

Predicted Amino Acid Sequence of Rat and Human MR77. The predicted protein contains seven hydrophobic regions as determined by hydropathy calculations (23) (data not shown). In addition, the N-terminal putative extracellular domain has two potential sites for asparagine-linked glycosylation at amino acids 5 and 10 (Fig. 1, black triangles). There are several potential protein kinase C (circles) and calmodulin kinase II phosphorylation (squares) sites in the large intracellular loop between putative transmembrane domains V and VI (Fig. 1). These potential posttranslational modifications are similar to those predicted for a variety of G-protein-coupled receptors (24, 25). MR77 exhibits considerable amino acid homology, particularly within the transmembrane domains, with known 5-HT receptors that are coupled to the inhibition of adenylate cyclase: human S31 (5-HT_{1E}), dog 5-HT_{1D}, rat 5-HT_{1B}, rat 5-HT_{1A}, and rat 5-HT_{1C} with 55%, 48%, 46%, 35%, and 30% overall identity. The primary amino acid sequence of MR77 is most closely related to the recently described human S31 receptor (26), which has been identified as the 5-HT_{1E} receptor (9). We screened a human genomic library with rat MR77 and determined the sequence of the human MR77 homolog, which is 86% and 93% identical to rat MR77 at the nucleic acid and amino acid sequence levels, respectively. The human amino acids that differ from those of rat are shown in italics in Fig. 1. The relationships among the known G-protein-coupled serotonin receptors are graphically illustrated as a dendrogram (Fig. 2). The 5-HT_{1B} and

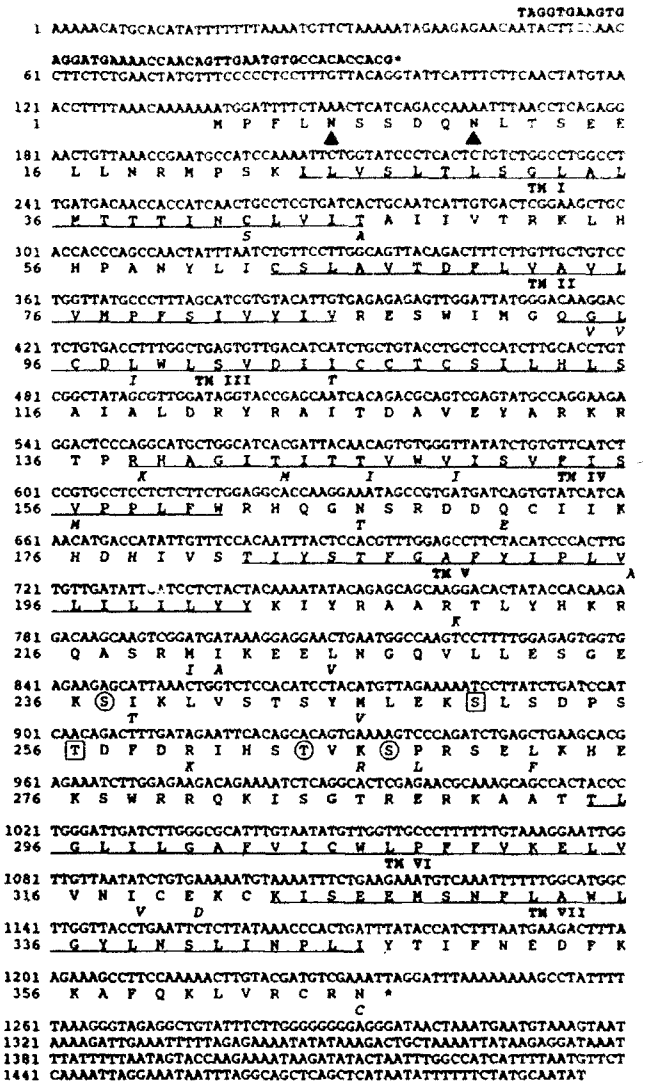

FIG. 1. Nucleotide sequence and predicted amino acid sequence of the rat MR77 genomic clone. The putative transmembrane domains are underlined. Triangles indicate potential N-linked glycosylation sites. The asterisk in line 2 is located above the intron splice junction. The sequence upsfream of this site in boldface print represents the MR77 cDNA sequence, which is different from the genomic clone. The cDNA sequence downstream from the asterisk is identical to the genomic clone (not shown). The asterisk at the end of the amino acid sequence signifies the termination codon. Circles and squares indicate amino acids that are consensus phosphorylation sites for protein kinase C and calmodulin kinase II, respectively. Amino acids in italics beneath the rat amino acid sequence represent differences with the human clone.

5-HT_{1D} receptors and the MR77 and 5-HT_{1E} receptors form distinct subfamilies that are more similar to one another than to other receptor types, as determined by amino acid sequence comparisons. In addition, the 5-HT_{1B}, 5-HT_{1D}, MR77, and 5-HT_{1E} receptors are more similar to each other collectively than to 5-HT_{1A}. This may be due in part to the longer putative third intracellular loop in 5-HT_{1A} compared with the other receptors.

Brain Regional Distribution of MR77 mRNA Expression. The tissue distribution of MR77 expression could not be determined by Northern blotting because of the low expression of the mRNA. However, we were able to take advantage of PCR and the fact that the MR77 gene contained an intron in its 5′ untranslated region. Primers were designed so as to amplify MR77, producing a 563-bp fragment from cDNA

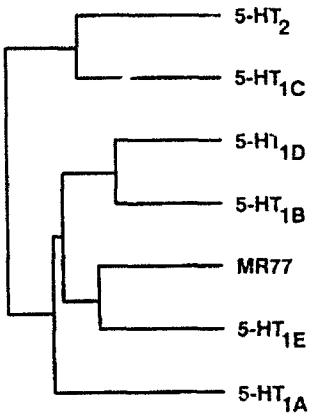

FIG. 2. Dendrogram of the G-protein-linked 5-HT receptor family based on sequence similarity. The relative lengths of the bars are inversely proportional to the similarity between the sequence pairs.

templates but not from genomic DNA (Fig. 3*A*). Therefore, detection of amplified product should be diagnostic for the presence of MR77 mRNA. Equal amounts of cDNA made from mRNA extracted from a variety of rat tissues were subjected to PCR, and a major band migrating at approximately 570 bp was observed with some templates (Fig. 3*B*). The 570-bp PCR product was subcloned, and its sequence was shown to be identical to the appropriate region of the

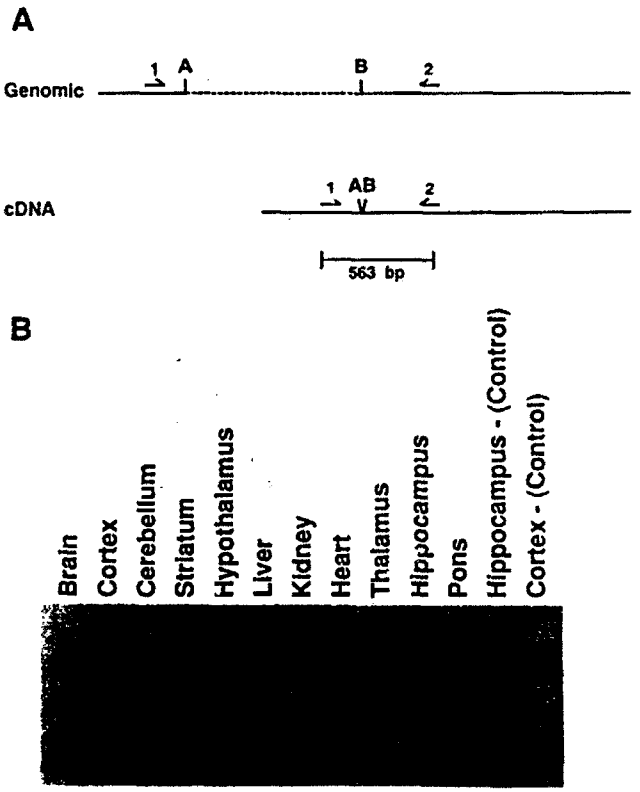

FIG. 3. PCR-based detection of MR77 mRNA expression. (*A*) Point B corresponds to nucleotide 97 of the MR77 genomic clone (Fig. 1). The distance between oligonucleotides 1 and 2 plus their lengths is 563 bp on the cDNA clone. The distance between point A and B on the genomic clone is not known. (*B*) The regional distribution of MR77 mRNA expression. cDNAs from the tissues listed were used as templates for the PCR reaction in *A*. The major band (middle band) migrates at a size approximating 570 bp. The blot was probed with an ^{32}P-labeled oligonucleotide corresponding to nucleotides 257–278 of the genomic clone. Control lanes 12 and 13 contained mRNA templates that had not been reverse-transcribed.

MR77 cDNA. The MR77 PCR product was detected in approximately the following abundance: cortex = striatum = hippocampus > thalamus = pons > hypothalamus > cerebellum. This distribution is consistent with known central nervous system terminal fields for serotonergic pathways. The abundance of MR77 mRNA in the cerebral cortex was estimated to be approximately 0.0001% of total mRNA, based on the comparison of the PCR product formed from cortex cDNA template to the product formed from known amounts of cloned MR77 DNA template. No PCR product was detected with liver, kidney, or heart cDNA templates. To show that the PCR products were not the result of amplification of genomic DNA, the same hippocampus and cortex mRNAs used to make cDNA template were additionally used as templates in PCR. These reactions did not yield any products (lanes 12 and 13, Fig. 3*B*).

Expression and Pharmacological Analysis of MR77. To determine the pharmacological profile of this receptor, we subcloned the gene into a eukaryotic expression vector (pCMV4) and transfected CosM6 cells. Broken cell preparations from transfected cells exhibited saturable ^{125}I-LSD binding that was competitively antagonized by 5-HT but not by either of the two catecholamines dopamine and norepinephrine. Saturation isotherm binding experiments demonstrated that ^{125}I-LSD binds with high affinity (K_d = 21.2 nM ± 5) to an apparent single class of noninteracting binding sites.

Table 1 shows the IC_{50} values for competition by a variety of selective and nonselective 5-HT ligands for ^{125}I-LSD binding to MR77 and the reported 5-HT$_{1E}$ receptor. MR77 demonstrated a high affinity for 5-HT, much like members of the 5-HT$_1$ subfamily. As predicted by the primary sequence analysis, MR77 exhibited a 5-HT$_{1D}$-like pharmacology in that it had affinity for sumatriptan but not 8-hydroxy-2(di-*n*-propylamino)tetralin. However, MR77 did not bind the mixed 5-HT$_{1A/1D}$ agonist 5-CT. MR77 also exhibited high affinity for the ergot alkaloids ergotamine and methysergide. ^{125}I-LSD binding to MR77 was relatively unaffected by the 5-HT$_{1C/2}$-selective ligands methiothepin and mesulergine. Thus, MR77 has a unique pharmacological profile, and its affinity for 5-HT classifies it as a member of the 5-HT$_1$ subfamily.

Table 1. Competition studies for ^{125}I-LSD-labeled sites on rat MR77 vs. 5-HT$_{1E}$ receptor affinity constants

Ligand	MR77 IC_{50}, nM ± SE	5-HT$_{1E}$ K_i, nM
Methysergide	14 ± 2	300
Ergotamine	55 ± 7	600
Sumatriptan	67 ± 3	2300
5-HT	70 ± 3	7
Metergoline	535 ± 43	1100
Yohimbine	590 ± 31	—
5-CT	2593 ± 662	7100
8-OH-DPAT	>1000	—
SKF 83566	>1000	—
Mesulergine	>1000	—
Dopamine	>1000	—
Norepinephrine	>1000	—
Methiothepin	>1000	200

CosM6 cells were transfected with an expression vector containing MR77 cDNA. Cell membranes were labeled with ^{125}I-LSD, which was competitively antagonized by a variety of ligands. IC_{50} values represent the concentrations of ligands (nM ± SEM) at which 50% of the bound ^{125}I-LSD could be displaced. 5-HT$_{1E}$ values are from McAllister *et al.* (9). 5-CT, 5-carboxyamidotryptamine; 8-OH-DPAT, 8-hydroxy-2-(di-*n*-propylamino)tetralin.

The insensitivity of MR77 to 5-CT was similar to that observed for the reported human 5-HT_{1E} receptor (12, 13). Leonhardt and coworkers (13) demonstrated that the putative 5-HT_{1E} receptor had high affinity for 5-HT, ergotamine, and methysergide but not 5-CT. Although the reported K_i values for ligand binding at the 5-HT_{1E} receptor were slightly different than those for MR77, the general trend was similar. McAllister *et al.* (9) have recently cloned DNA for a human receptor that was identical to the *S31* gene and have reported that the pharmacology of the receptor matches exactly that for the putative 5-HT_{1E} receptor (13). Table 1 lists the K_i values for competition binding vs. tritiated 5-HT in cells transfected with the S31 receptor gene. The 5-HT_{1E} receptor is insensitive to sumatriptan, whereas MR77 displays significant affinity for this ligand. In addition, the ergot alkaloids methysergide and ergotamine display lower affinity for the 5-HT_{1E} receptor than for MR77. It should be noted that our binding assays used ^{125}I-LSD, whereas the 5-HT_{1E} studies used tritiated 5-HT. These different agents may result in the variations between affinity constants determined for the ligands tested.

Inhibition of Adenylate Cyclase. The primary structure of MR77 suggested that this receptor may be coupled to the inhibition of adenylate cyclase through an inhibitory G-protein (G_i)-like protein. This assumption was based on similarities among G_i-coupled receptors, which tend to have longer intracellular loops between transmembrane regions five and six as well as shorter C-terminal tails compared with stimulatory G-protein (G_s)-coupled receptors. We tested whether MR77 could mediate the inhibition of adenylate cyclase induced by serotonin. In cells cotransfected with MR77 and the hamster β_2-adrenergic receptor, 10 μM 5-HT elicited a 32 $\pm$ 6% (n = 3) decrease of isoproterenol-stimulated cAMP accumulation. Cells cotransfected with the rat 5-HT_{1A} receptor, known to couple to G_i, and the hamster β_2-adrenergic receptor exhibited a 53 $\pm$ 9% (n = 3) reduction in cAMP accumulation in response to 10 μM 5-HT. Cells transfected with the hamster β_2-adrenergic receptor alone showed almost no inhibition (8 $\pm$ 6%) in response to 10 μM 5-HT. These results show that MR77 is capable of coupling in an inhibitory manner to adenylate cyclase, presumably through a G_i-like mechanism.

CONCLUSION

The data presented demonstrate that the MR77 gene encodes a 5-HT receptor protein that is expressed predominantly in the central nervous system. The mRNA distribution pattern in the brain is consistent with terminal fields for some serotonergic neurons. MR77 couples to the inhibition of adenylate cyclase and has a binding profile similar to that for the 5-HT_{1D} receptor with the marked exception in affinity for 5-CT. Interestingly, sumatriptan and ergotamine, both of which exhibit high affinity for MR77, have been shown to have clinical efficacy for the treatment of migraine headaches (for review, see ref. 27). This raises the possibility that the action of these drugs may be mediated by more than one subtype of 5-HT receptors.

Lately, the identification of receptor genes has outpaced traditional pharmacological identification. Therefore, it has become increasingly difficult to classify receptor gene products on a basis other than amino acid sequence. The pharmacological profile of the MR77 receptor protein is similar to the previously identified 5-HT_{1E} receptor with regard to the apparent insensitivity to the ligand 5-CT but differs with regard to the affinity for sumatriptan. Given that the sequence of MR77 is most similar to 5-HT_{1E}, we propose that the MR77 gene be tentatively identified as another member of the 5-HT_{1E} receptor subgroup.

Note Added in Proof. The mouse homolog of this 5-HT_{1E}-like receptor has been reported (28).

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